THE SUBFRACTIONATION OF PLATELET MEMBRANES BY ZONAL CENTRIFUGATION: IDENTIFICATION OF SURFACE MEMBRANES

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1. Introduction

Several subcellular fractionation procedures have been described for separating platelet organelles from homogenates prepared in isotonic sucrose. These fractionations usually produce a soluble phase, a low density particulate fraction containing membrane vesicles, and a heavier, granular fraction containing the mitochondria, amine-storage bodies and the lysosome-like organelles [1–4]. The platelet membrane fractions so prepared, although essentially free of granular organelles, are generally still heterogenous since they are derived probably from both the outer membrane of the cell and the extensive complex of intracellular membranes that platelets are known to contain [5].

In this communication, it is shown that platelet membrane fractions prepared by tube sucrose density gradient centrifugation can be separated further into two subfractions of membrane vesicles by zonal gradient centrifugation in the 'B 14' titanium rotor. This study also shows that each of these membrane subfractions has associated with it one of two distinct phosphodiesterase activities, both of which are identifiable in the original membrane fraction. Further, by labelling the platelet surface before homogenisation, using a ^{125}iodide-labelled anti-platelet membrane antibody, and also by iodination using a lactoperoxidase–glucose oxidase procedure [6], it has been demonstrated that the lower density membrane subfraction contains surface-derived components. This fraction is rich in a phosphodiesterase activity upon bis-(*p*-nitrophenyl) phosphate and the studies indicate that this enzyme activity may have considerable value as a platelet surface membrane marker.

2. Experimental

The procedure for the isolation of platelets from freshly collected pig blood, and their homogenisation in 0.3 M sucrose (buffered with 5 mM Tris–HCl, pH 7.4) using a M.S.E. top-drive homogeniser, has been described earlier by Harris and Crawford [2]. Their method for subcellular fractionation of the homogenates has been modified slightly as follows:

Platelet homogenate (ca. 12 ml) was layered onto sucrose gradients prepared in 35 ml M.S.E. polycarbonate tubes by successively layering 1 ml of 2.0 M sucrose, 4 ml each of 1.4 M, 1.2 M, 1.0 M, 0.8 M and 0.6 M sucrose and finally 2 ml of 0.4 M sucrose. All sucrose solutions were buffered with 5 mM Tris–HCl, pH 7.4. The tubes were centrifuged in the M.S.E. 3 × 40 ml swing out rotor at 50 000 gav for 3 hr at 4°C.

The membrane fractions from these gradient separations (upper particulate zone) were pooled, diluted 2:1 with 5 mM Tris–HCl, pH 7.4, and a 50 ml aliquot was applied to a 500 ml linear gradient of 18–36% w/w sucrose (pH 7.4) in the M.S.E. titanium 'B 14' zonal rotor. A cushion of 60% w/w sucrose and an overlay of 40 ml 5% w/w sucrose were used. The rotor was centrifuged at 47 000 rev/min for 18 hr at 4°C, and 20 ml fractions were collected.

For the immunochemical labelling, a gamma-globulin fraction was prepared as described by Nairn [7]

from a sheep antiserum raised to pig platelet membranes prepared by the tube gradient fractionation. This γ-globulin was labelled with ^{125}iodide using a chloramine-T procedure [8]. Whole platelets were then incubated with the labelled antibody for 5 min at 20°C, in the proportion of 1 g platelets (wet wt.) to 1 mg γ-globulin. The incubation medium contained 0.154 M NaCl, 0.154 M Tris–HCl (pH 7.4) and 0.077 M EDTA in a volume ratio of 90:8:2. These labelled cells were then washed free of unbound antibody and mixed with unlabelled platelets suspended in 0.3 M sucrose, in a ratio of 1:4, prior to homogenisation.

The procedure for the lactoperoxidase-catalysed iodination of the platelet surface membrane with ^{125}iodide was essentially that described by Nachman et al. [6]: these labelled cells were also mixed in a ratio of 1:4 with unlabelled platelets prior to homogenisation.

Radioactive samples were counted in type 'B' Gammavials (Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K.) using the tritium channel of a Nuclear Chicago 'Isocap/300' liquid scintillation system.

The homogenates and the tube and zonal gradient membrane fractions were assayed for the following enzymes: phosphodiesterase (PDE) acting on bis-(*p*-nitrophenyl) phosphate at pH 5.5 [9], phosphodiesterase activity towards 5'-thymidine-*p*-nitrophenyl phosphate at pH 7.9 (both these PDE activities were determined by *p*-nitrophenol liberation), *N*-acetyl-β-glucosaminidase [10] and succinate dehydrogenase [11]. Protein was determined by the method of Lowry et al. [12]; zonal fractions were diluted 1:10 to overcome the interference by sucrose. Sucrose densities were measured at 0°C using an Abbé refractometer.

The membrane subfractions were dialysed extensively against 1 mM Tris–HCl, pH 7.4 to remove sucrose, and extracted with chloroform:methanol (2:1 v/v) according to Garbus et al. [13]. The chloroform extract was used for cholesterol estimation [14] and a perchloric acid digest of it was used for phospholipid phosphorus determination [15].

For electron microscopy the membrane fractions were diluted 1:1 with 5 mM Tris–HCl, pH 7.4, and centrifuged at 100 000 *gav* for 60 min. The pellets were suspended in 6.25% (v/v) glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) for 30 min, washed in cacodylate buffer for 1 hr, suspended in 1% (w/v) osmium tetroxide in 0.285 M barbitone sodium–sodium acetate–HCl buffer (pH 7.4) for 2 hr, washed in barbitone–acetate buffer for 1 hr, dehydrated through alcohol and propylene oxide and embedded in Araldite. Ultra-thin sections were stained with uranyl acetate (saturated solution in methanol) for 15 min, and viewed with a Philips model 301 electron microscope.

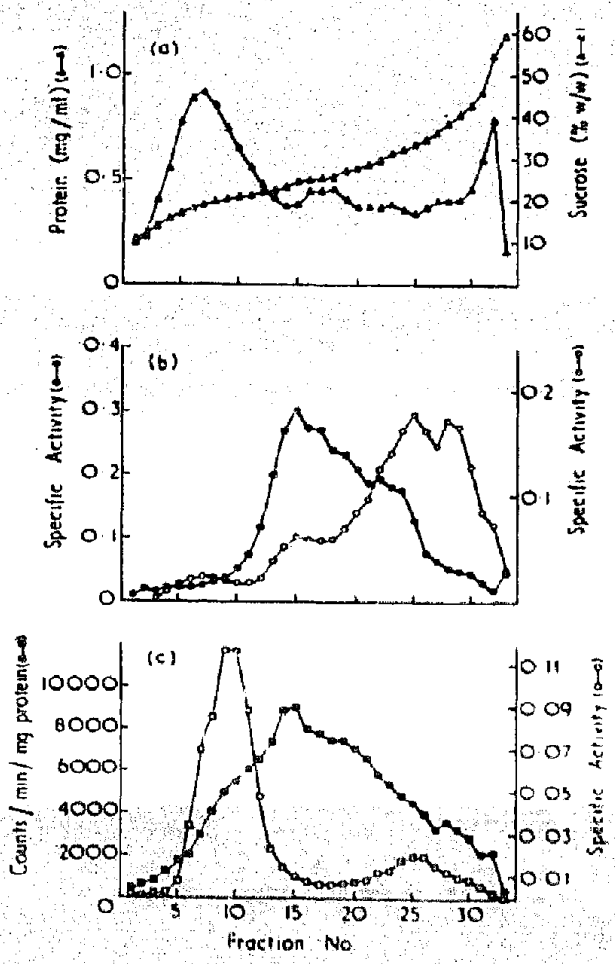

Fig. 1. Subfractionation of platelet membranes, labelled with ^{125}I iodide antibody, by centrifugation in the 'B 14' zonal rotor. Enzyme profiles expressed as specific activity (μmoles product released/hr/mg protein). (a) ▲, protein ○, sucrose gradient; (b) ●, bis-(*p*-nitrophenyl) phosphate PDE; ○, 5'-thymidine-*p*-nitrophenyl phosphate PDE; (c) ■, ^{125}I iodide (counts/min/mg protein); □, *N*-acetyl-β-glucosaminidase.

Table 1
A comparison of enzyme activities in pig platelet homogenate (HOM), the membrane fraction (MEM) prepared by tube density gradient centrifugation and the membrane subfractions (I & II) prepared by zonal centrifugation.

	Specific activities			
	HOM	MEM	Subfraction I	Subfraction II
Bis-(*p*-nitrophenyl) phosphate PDE	0.110	0.299	0.688	0.202
5' Thymidine-*p*-nitrophenyl phosphate PDE	0.048	0.147	0.082	0.404
N-Acetyl-β-glucosaminidase	0.057	0.037	0.007	0.020
Succinate dehydrogenase	0.050	0.002	0	0

The results are expressed as μmoles product released/hr/mg protein.

3. Results

After tube density gradient centrifugation of platelet homogenates the membrane fraction located in the upper of the two major particulate zones at a density range of 1.100–1.150. (This corresponds to zone B of the Harris and Crawford procedure [2].) This membrane fraction is low in acid hydrolases and mitochondrial marker enzyme activities (table 1) but has high levels of the two phosphodiesterase activities, each showing 3–4 fold increases in specific activity relative to the homogenate.

When this membrane fraction was subjected to further centrifugation in the 'B 14' zonal rotor the two phosphodiesterase activities were then resolved clearly from each other as illustrated in fig. 1. The peak of the bis-(*p*-nitrophenyl) phosphate PDE activity (membrane subfraction I) located in the range 24–25% w/w sucrose (density) 1.105–1.110) and the 5'-thymidine-*p*-nitrophenyl phosphate PDE peak (subfraction II) occurred in the range 32–33% w/w sucrose (density 1.140–1.150). The two PDE activities at their respectivespeaks each showed 2–3–fold purification with respect to the whole membrane fraction (table 1) and 6–8-fold purification relative to the homogenate.

The distribution of *N*-acetyl-β-glucosaminidase activity, which we found to be representative of several platelet glycosidases, indicates that the activity of this enzyme in the whole membrane fraction is derived probably from residual soluble phase material, since there was negligible activity associated with the two subfractions. There was no detectable succinate dehy-

Table 2
Cholesterol and phospholipid content of platelet membrane subfractions

	Subfraction I	Subfraction II
μmoles Cholesterol/mg protein (Range)	0.516 (0.492–0.535)	0.203 (0.118–0.292)
μmoles Phospholipid/mg protein (Range)	0.668 (0.626–0.732)	0.274 (0.153–0.389)

The values are the means of duplicate assays from three separate fractionations.

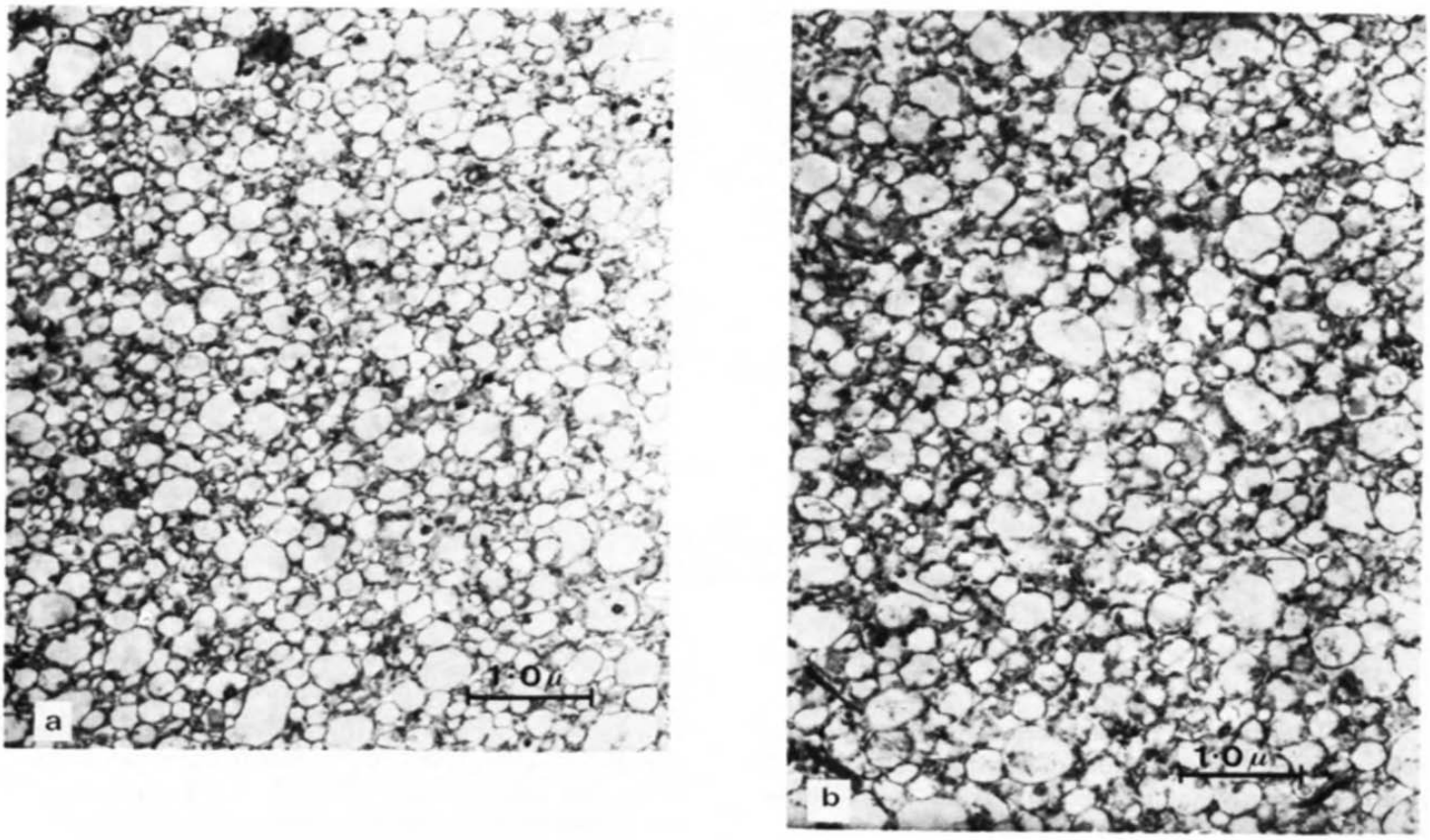

Fig. 2. Electron micrographs (× 14 000) of glutaraldehyde, osmium tetroxide fixed sections of platelet membrane subfractions. (a) subfraction I; (b) subfraction II.

drogenase activity in the zonal fractions.

Electron micrographs (fig. 2) of the fractions corresponding to the peaks of the two PDE activities showed that both subfractions consisted of small membrane vesicles with no obvious morphological differences between them, and the original membrane fraction.

The data in fig. 1 has been taken from an ^{125}iodide-γ-globulin labelling experiment, and it can be seen that the distribution of the ^{125}iodide label followed closely that of the bis-(*p*-nitrophenyl) phosphate PDE activity.

The results of a similar experiment, using a platelet homogenate in which approx. 20% (w/w) of the cells had been exposed to the lactoperoxidase-catalysed iodination procedure, are presented in fig. 3. It is clear that the two phosphodiesterase activities were again well resolved and located in the same density ranges as in the previous experiment. The distribution of this ^{125}iodide label also coincided closely with the distribution of the bis-(*p*-nitrophenyl) phosphate PDE activity.

The results of the determinations of the cholesterol, phospholipid and protein content of the two subfractions are presented in table 2. It is apparent that subfraction I contains more than twice as much cholesterol and phospholipid per unit weight of protein than subfraction II, although both subfractions have a similar molar ratio of cholesterol to phospholipid. This difference in the lipid content of the two subfractions probably accounts for their different buoyant densities.

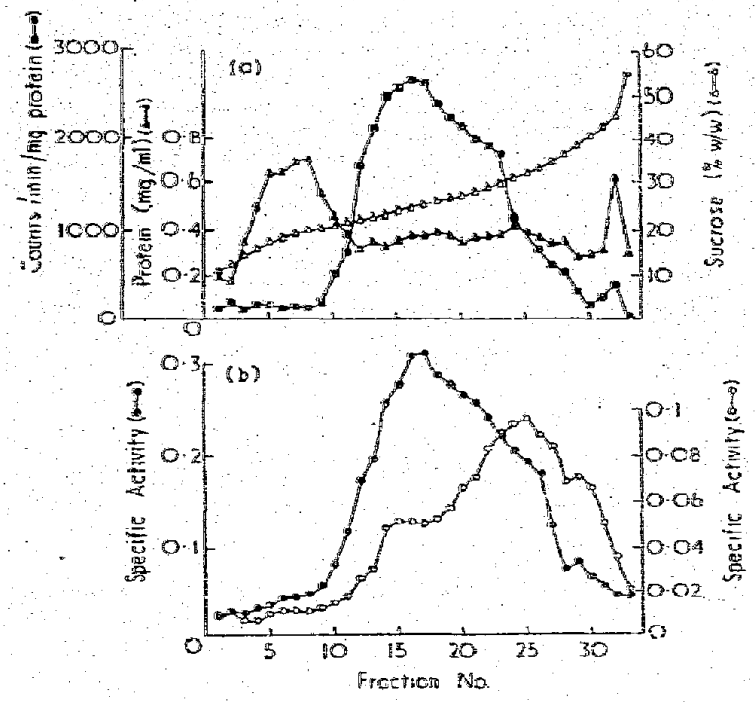

Fig. 3. Zonal subfractionation of platelet membranes labelled with ^{125}iodide by the lactoperoxidase procedure. Enzyme profiles expressed as specific activity (μmoles/hr/mg protein). (a) ▲, protein; △, sucrose gradient; ■, ^{125}iodide (counts/min/mg/protein); (b) ●, bis-(*p*-nitrophenyl) phosphate PDE; ○, 5′-thymidine-*p*-nitrophenyl phosphate PDE.

4. Discussion

The platelet, in addition to a surface membrane, contains an extensive complex of intracellular membrane structures [5] and although membrane fractions prepared from platelet homogenates by a single, short duration tube density gradient procedure contain surface membranes, fragments from the internal membrane structures are almost certainly also present. Studies of the distribution of surface and intracellular membranes in platelet subcellular fractions have been complicated by the absence of the well defined marker enzyme activities which have proved useful in fractionations of other cells [2].

The heterogeneity of platelet membrane fractions prepared by tube density gradient methods is illustrated by the isolation of two distinct subfractions by zonal centrifugation as described here. The basis of this subfractionation lies in the separation of two different phosphodiesterase activities, both of which show high specific activities in the whole membrane fraction with respect to the homogenate. The results of the surface membrane labelling experiments using both the ^{125}iodide-labelled γ-globulin fraction and the lactoperoxidase iodination procedure support strongly our view that the low density membrane subfraction (I), possessing high specific activity for bis-(*p*-nitrophenyl) phosphate phosphodiesterase, is derived mainly from the surface membrane of the cell. Consequently it would appear that this phosphodiesterase is a valuable marker enzyme for the future study of surface-derived components of platelets.

Barber and Jamieson [16] have separated two membrane fractions from an homogenate of human platelets prepared by a glycerol-loading technique. However, both fractions showed high relative specific activities for bis-(*p*-nitrophenyl) phosphate PDE and since both were labelled by a lactoperoxidase iodination procedure [17], it would appear that these two membrane fractions contained surface membrane elements.

The precise origin of the higher density membrane subfraction (II) rich in 5′-thymidine-*p*-nitrophenyl phosphate phosphodiesterase activity, obtained by the zonal fractionation described here, has not been established although it is reasonable to conclude that it is derived from intracellular membranes. The naturally occurring substrates for the two phosphodiesterase activities used in this study have yet to be determined.

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References

[1] Minter, B. F. and Crawford N. (1971) Biochem. Pharmacol. 20, 783–802.
[2] Harris, G. L. A. and Crawford, N. (1973) Biochim. Biophys. Acta 291, 701–719.
[3] Day, H. J., Holmsen, H. and Hovig, T. (1959) Scand. J. Haematol. Suppl. 7, 1–35.

[4] Marcus, A. J., Zucker-Franklin, D., Safier, L. B. and Ullman, H. L. (1966) J. Clin. Invest. 45, 14–28.
[5] White, J. G. (1972) Amer. J. Pathol. 66, 295–312.
[6] Nachman, R. L., Hubbard, A. and Ferris, B. (1973) J. Biol. Chem. 248, 2928–2936.
[7] Nairn, R. C. (1969) in: Fluorescent Protein Tracing, 3rd edition, p. 303, Livingstone Ltd., London.
[8] McConahey, P. J. and Dixon, F. J. (1966) Int. Arch. Allergy 29, 185–189.
[9] Koerner, J. F. and Sinsheimer, R. L. (1957) J. Biol. Chem. 228, 1039–1048.
[10] Robinson, D., Price, R. G. and Dance, N. (1967) Biochem. J. 102, 525–532.
[11] Pennington, R. J. (1961) Biochem. J. 80, 649–654.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[13] Garbus, J., Deluca, H. F., Loomans, M. E. and Strong, F. M. (1963) J. Biol. Chem. 238, 59–63.
[14] Crawford, N. (1958) Clin. Chim. Acta 3, 357–367.
[15] Chalvardjian, A. and Rudnicki, E. (1970) Analyt. Biochem. 36, 225–226.
[16] Barber, A. J. and Jamieson, G. A. (1970) J. Biol. Chem. 245, 6357–6365.
[17] Baroer, A. J. and Jamieson, G. A. (1971) Biochemistry 10, 4711–4717.